SUMMARY

Mitochondria constantly respond to changes in substrate availability and energy utilization to maintain cellular ATP supplies, and at the same time control reactive oxygen radical (ROS) production. Reversible phosphorylation of mitochondrial proteins has been proposed to play a fundamental role in metabolic homeostasis, but very little is known about the signaling pathways involved. We show here that protein kinase A (PKA) regulates ATP production by phosphorylation of mitochondrial proteins, including subunits of cytochrome c oxidase. The cyclic AMP (cAMP), which activates mitochondrial PKA, does not originate from cytoplasmic sources but is generated within mitochondria by the carbon dioxide/bicarbonate-regulated soluble adenylyl cyclase (sAC) in response to metabolically generated carbon dioxide. We demonstrate for the first time the existence of a CO2-HCO3-/C02-sAC-cAMP-PKA (mito-sAC) signaling cascade wholly contained within mitochondria, which serves as a metabolic sensor modulating ATP generation and ROS production in response to nutrient availability.

INTRODUCTION

The Krebs Cycle (TCA cycle) produces the electron donors, which drive mitochondrial production of ATP via oxidative phosphorylation (OXPHOS). OXPHOS is subject to complex regulation, including short-term modulations essential for responding to transient changes in nutritional availability, environmental conditions, and energy requirements. If the reducing equivalents generated by the TCA cycle are not efficiently utilized by the OXPHOS machinery, reactive oxygen species (ROS) production may increase, and oxidative damage may ensue. It has been proposed that dynamic protein phosphorylation plays a major role in these rapid modulations (Hopper et al., 2006).

Evidence has emerged suggesting that cyclic AMP (cAMP)-mediated phosphorylation of mitochondrial enzymes plays a role in OXPHOS regulation. Consistent with this hypothesis, both protein kinase A (PKA) (reviewed in Pagliarini and Dixon, 2006; Thomson, 2002) and A kinase-anchoring proteins (AKAPs) have been identified in mammalian mitochondria (Felicelli et al., 2005; Lewitt et al., 2001). In particular, PKA in the mitochondrial matrix has been demonstrated by several independent groups using biochemical, pharmacological, and immunological methods, including immunoelectron microscopy (Livigni et al., 2006; Prabu et al., 2006; Ryu et al., 2005; Schwoch et al., 1990). However, if PKA plays a role in phosphorylating mitochondrial proteins, it remains unclear how the cAMP that activates PKA is modulated. Specifically, cAMP does not diffuse far from its source (Bornfeldt, 2006; Zaccolo and Pozzan, 2002), and as we show here, it does not enter mitochondria. Papa et al. postulated that a source of this second messenger might reside inside mitochondria (Papa et al., 1999), but an intramitochondrial adenylyl cyclase had not been demonstrated so far.

In mammalian cells, cAMP can be produced by a family of plasma membrane-bound forms of adenylyl cyclase (tmAC), or by a “soluble” adenylyl cyclase (sAC) (Buck et al., 1999). We previously showed that sAC resides at multiple subcellular organelles, including mitochondria (Zippin et al., 2003). Unlike tmACs, sAC is insensitive to heterotrimeric G protein regulation or forskolin; instead, it is stimulated by bicarbonate (Chen et al., 2000) and sensitive to ATP (Litvin et al., 2003) and calcium levels (Jaiswal and Conti, 2003; Litvin et al., 2003). Bicarbonate stimulates sAC activity by facilitating active site closure, while calcium promotes activity by increasing the affinity for ATP (Litvin et al., 2003; Steegborn et al., 2005). In physiological systems, including mitochondria (Dodgson et al., 1980), carbonic anhydrases (CA) convert CO2 into bicarbonate. While generating electron donors for OXPHOS, the TCA cycle generates CO2 and therefore bicarbonate. Thus, sAC represents an excellent candidate OXPHOS regulator, which ensures that respiration can keep pace with changes in nutritional availability and prevent ROS accumulation.

Here we show that PKA modulation of OXPHOS activity is regulated by cAMP generated inside mitochondria by sAC in response to metabolically generated CO2. This study provides a functional understanding of the modulation of OXPHOS in direct response to nutrient metabolism by the mito-sAC signaling pathway.

RESULTS

cAMP-PKA Regulation of OXPHOS

To test whether mitochondrial OXPHOS can be regulated by PKA, we stimulated HeLa cells with membrane-permeant agents that cause a transient rise in cAMP levels.
mt-cAMP Regulates OXPHOS

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mt-cAMP, which activates all cAMP-dependent kinases. We measured oxygen consumption as an indicator of mitochondrial respiratory chain function. 8Br-cAMP (1 mM for 30 min) resulted in a 25% (p < 0.0001) increase in oxygen consumption, as compared to untreated cells (Figure 1A). Then, we uncoupled oxygen consumption from ATP synthesis by inclusion of carbonic anhydrase 4–(trifluoromethoxy)-phenylhydrazine (FCCP). Under these conditions, where respiratory chain activity is independent from ATP synthesis by the F$_{1}$/F$_{0}$ ATPase, 8Br-cAMP still increased oxygen consumption. Note that the residual ATP content in mitochondria treated with FCCP for 5 min was approximately 60% of the pre-FCCP content (2.5 ± 0.3 and 4.1 ± 0.2 nmol/mg protein, respectively). Thus, there still was sufficient ATP for phosphorylation of PKA target proteins. The exchange protein activated by cAMP-selective agonist, 8CPT methyl-cAMP, did not change coupled or uncoupled respiration.

Conversely, H89 at 1 μM, a concentration that selectively blocks PKA, resulted in a 50% decrease in coupled and uncoupled respiration. RpcAMP (25 μM) which inhibits PKA by a different mechanism, also caused a similar decrease (20%, p < 0.001) in oxygen consumption (data not shown). The effects of PKA agonists and antagonists were replicated in 143B human osteosarcoma and 293T-HEK (data not shown), except that the increase in respiration induced by FCCP was higher (i.e., approximately 100% in 143B and 293T compared to 20% in HeLa cells), reflecting different coupling between oxygen consumption and ATP synthesis.

Stimulation of tmAC with forskolin in combination with the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) did not affect mitochondrial respiration, despite an 8- to 10-fold increase in cytoplasmic cAMP (Figure 1B). Therefore, mitochondrial respiration is enhanced through PKA activation by membrane-permeant cAMP analogs, but not by cytoplasmic cAMP, suggesting that the PKA that modulates respiration is inside mitochondria. It was suggested that tmAC-generated cAMP enters the mitochondrial matrix. However, our data demonstrate that cytoplasmic cAMP does not have access to the intramitochondrial PKA pool. DiPilato and colleagues used a reporter protein targeted to mitochondria, but the mitochondrial import was partial, with a portion of the protein remaining on the cytosolic surface or in the mitochondrial intermembrane space (DiPilato et al., 2004), where it remained accessible to cytosolic cAMP.

To further explore the role of intramitochondrial PKA in modulating OXPHOS, we examined isolated mitochondria from mouse liver.

First we measured state III (phosphorylating) respiration driven by different substrates: glutamate/malate (G + M) specific for complex I (Figure 1C), succinate for complex II (Figure 1D), and TMPD + ascorbate for complex IV (Figure 1E). Similar to intact cells, 8Br-cAMP produced a small but significant increase in respiration with glutamate/malate (12%, p < 0.05) and TMPD/ascorbate (13%, p < 0.05). Succinate-dependent respiration was unchanged, indicating that it cannot be upregulated by cAMP. As expected, addition of exogenous, membrane-impermeant cAMP, or 8CPT methyl-cAMP, or forskolin + IBMX had no effect on oxygen consumption (Figures 1C–1E). Similar to whole cells, the PKA inhibitor, H89, decreased oxygen consumption driven by all complexes (44% for glutamate/malate, p < 0.0001; 50% for succinate, p < 0.0001; 30% for TMPD/ascorbate, p < 0.0001). Another PKA-specific inhibitor, myristoylated PKI 14-22, also inhibited respiration (20% for glutamate/malate, p < 0.001; 36% for succinate, p < 0.001; 25% for TMPD/ascorbate, p < 0.0001). 8Br-cAMP was inert in the presence of PKI 14-22, confirming the role of PKA in modulating respiration.

Second, we showed that ATP synthesis (Figure 1F) was also enhanced by 8Br-cAMP (75%, p < 0.0001; inhibited by H89 and PKI 14-22 (63% and 74%, respectively, p < 0.0001), and unchanged by membrane impermeant cAMP.

Third, the capacity to generate mitochondrial membrane potential (ΔΨm) was measured fluorimetrically under nonphosphoryling conditions. Figure 1G shows representative fluorescence traces. 8Br-cAMP increased membrane potential driven by glutamate/malate or succinate (21% ± 8% and 29 ± 6%, respectively; p < 0.01), whereas H89 decreased it (37% ± 8% and 51% ± 10%, respectively; p < 0.0001).

Because PKA inhibitors decreased oxygen consumption independent of which electron transfer complex was stimulated (Figures 1C–1E), the modulating effect of PKA likely targets COX, the terminal component of the respiratory chain. Consistently, COX activity (rate of oxidation of reduced cytochrome c) was stimulated by 8Br-cAMP (25%, p < 0.0001; Figure 1H), but not by cAMP, 8CPT methyl-cAMP, or forskolin + IBMX, and was inhibited by H89 and PKI 14-22 (22% and 18%, respectively; p < 0.0001). The stimulation by 8Br-cAMP was blocked by PKI 14-22.

There were no changes in steady-state protein levels of COX subunits I and IV after modulation of PKA (Figure S1), suggesting that the activity changes depend on posttranslational modification of the enzyme kinetics. Consistently, it was previously proposed that COX is regulated via protein phosphorylation.
As expected for a short-term adaptation mechanism, the cAMP-induced changes in PKA modulation of COX activity were transient and readily reversible upon washout of the agonist (Figure S2).

sAC Is a Source of cAMP in Mitochondria

Membrane-impermeant cAMP had no effect on OXPHOS response, suggesting that a source of cAMP must reside within mitochondria. We previously showed that sAC immunoreactivity colocalizes and sAC activity copurifies with mitochondria (Zippin et al., 2003). We now show that western blotting using the R21 monoclonal anti-sAC antibody (Zippin et al., 2003) identifies multiple bands in the liver homogenate (Figure 2A), consistent with multiple sAC splice isoforms (Buck et al., 1999; Farrell et al., 2008; Geng et al., 2005; Jaiswal and Conti, 2003). However, purified mitochondria contained only one sAC isoform of approximately 48kDa (Figure 2A, lane 4). This band was confirmed in mitochondria highly purified by a second gradient step (Figure 2A, lanes 4.4). Instead, ER-rich fractions contained a distinct sAC isoform migrating at approximately 35kDa (Figure 2A, lanes 2 and 3). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was absent from the mitochondrial and ER fractions excluding detectable contamination with cytoplasmic proteins.

A highly active isoform of rat sAC (sACt) (Chaloupka et al., 2006) containing an N-terminal HA tag was expressed in COS cells. In cell homogenates (Figure 2B, lane 1) sACt was detected both by the R21 and HA antibodies. The crude mitochondrial fraction contained the same immunoreactive sACt bands (Figure 2B, lane 2). Likewise, endogenous PKA was detected in the mitochondrial fraction (Figure 2B, lane 2). A proteinase K protection assay performed on the mitochondrial fraction showed that portions of sACt and endogenous PKA were resistant to digestion, indicating that they resided in a protected mitochondrial compartment (Figure 2B, lane 3). Detergent solubilization of mitochondria allowed for complete digestion of sACt, PKA, as well as hsp60 and Tim23, localized in the mitochondrial matrix and inner membrane, respectively (Figure 2B, lane 4).

sAC is stimulated by bicarbonate (Chen et al., 2000), and adenylyl cyclase activity in mouse liver mitochondria demonstrated a significant bicarbonate stimulation (Figure 2C, p < 0.0001) and inhibition by the sAC-specific inhibitor, KH7 (Hess et al., 2005),
indicating that the mitochondrial component of sAC is enzymatically active.

The reversibility of the cAMP signal predicts that phosphodiesterase (PDE) should also be contained inside mitochondria. To test this hypothesis we isolated mitoplasts (i.e., mitochondria stripped of their outer membrane) from mouse liver. Mitoplasts contained inner membrane and matrix proteins (COX I, and Hsp60, respectively), but no intermembrane space or outer membrane proteins (Cyt c and Tom40, respectively), whereas the postmitoplast supernatants only contained Cyt c and Tom 40 (Figure 2D). After the contents of the matrix were made accessible by sonication, they degraded exogenous cAMP (Figure 2E). This cAMP catabolic activity was fully inhibited by IBMX, confirming the presence of intramitochondrial PDE activity.

Modulation of Intramitochondrial sAC Regulates OXPHOS

We used two distinct methods of increasing intramitochondrial sAC-generated cAMP to demonstrate a functional role for sAC in modulating OXPHOS activity.

First, sACt was stably overexpressed in 293T-HEK cells, and was found in both the whole cell homogenate and in isolated mitochondria (Figure 3A, lanes hom and 4, respectively). sACt overexpression increased respiration by approximately 25% (Figure 3B, p < 0.0001), as compared to untransfected cells. Consistently, COX activity (Figure 3C) and ATP synthesis (Figure 3D) were also increased (by 28% and 87%, respectively; p < 0.0001). These effects were antagonized by the sAC-specific inhibitor, KH7.

Second, we stimulated endogenous mouse liver sAC in isolated mitochondria with bicarbonate. We found that maximum stimulation of COX activity occurred at 30 mM bicarbonate (Figure 4A), which is consistent with the physiological intramitochondrial bicarbonate concentration (ranges between 10 and 40 mM) (Simpson and Hager, 1979). As predicted, bicarbonate enhanced ATP synthesis by 32% (Figure 4B, p < 0.001), and bicarbonate-dependent stimulation of COX activity at least partially accounted for this effect (18%) (Figure 4C, p < 0.001).

To confirm that bicarbonate stimulates OXPHOS, isolated mitochondria were exposed to the CO2-generating combination of z-ketoglutarate dehydrogenase complex (KGDHC), its substrates ketoglutaric acid + NAD+, and its cofactors coenzyme-A and cocarboxylase. KGDHC stimulated COX-driven respiration by 32%, using TMPD/ascorbate as substrates (Figures 4D and 4E, p < 0.0001). Addition of the carbonic anhydrase inhibitor (CAI) acetazolamide diminished COX driven respiration by 10% (p < 0.01), indicating that CO2 diffusing through the mitochondrial membranes is converted into bicarbonate that activates sAC and stimulates COX.

We note that, since OXPHOS activity is increased by both bicarbonate and by exogenously generated CO2, in a carbonic anhydrase-dependent manner, these effects cannot be due to pH changes (or ionic strength), because bicarbonate addition increases pH while CO2 addition decreases it.

To further demonstrate the role of sAC in modulating OXPHOS activity, we used three independent methods of blocking intra-mitochondrial sAC.

First, KH7, but not an inactive congener, KH7.15 (Wu et al., 2006), inhibited state III respiration driven by complexes I, II, and IV by 25%, 22%, and 35%, respectively (Figure 1C–1E, p < 0.0001). Inhibition of sAC by KH7 also markedly decreased ATP synthesis (80%) (Figure 4B, p < 0.0001), KH7, but not KH7.15, inhibited COX activity by 30% (Figure 4C, p < 0.0001). In each case, KH7 inhibition was rescued by membrane permeable 8Br-cAMP. Finally, KH7 decreased ΔΨm in isolated mitochondria by 46% ± 6% with glutamate/malate and by 62% ± 11% with succinate (Figure 1G, p < 0.0001). The effects of sAC
Figure 4. CO₂-TCA and sAC Modulation Regulates OXPHOS

(A) COX Vmax in isolated mitochondria treated with increasing concentration of HCO₃⁻, from 5 mM to 40 mM (n = 6).

(B and C) Isolated mitochondria from mouse liver incubated with bicarbonate (HCO₃⁻) (n = 4), or NaCl (n = 3) or KH7, (n = 9), KH7.15, (n = 3), KH7 + 8Br-cAMP (n = 3). ATP synthesis (B) was increased by HCO₃⁻ treatment, whereas KH7 inhibited it. COX Vmax was increased by HCO₃⁻, whereas KH7 diminished it (C). COX Vmax decrease was not observed with KH7.15, and was rescued by 8Br-cAMP.

(D) A representative trace of respiration driven by COX using TMPD/ascorbate mouse liver mitochondria. AA is added to mitochondria before TMPD/ascorbate to block electron transfer upstream of COX. KCN is added at the end to inhibit COX. Each addition is marked by downward arrowheads.

(E) Quantification of the experiments shown in D (n = 9).

(F) KH7 (n = 6) reduced respiration in intact HeLa cells, and this inhibition was prevented by 8Br-cAMP (n = 3), but not by forskolin + IBMX (n = 3). Control, untreated cells (n = 33).

(G) COX activity in intact or sonicated mitochondria with or without the inhibitory anti-sAC antibody R21. HCO₃⁻ stimulation of COX was antibody insensitive in intact mitochondria, whereas it was abolished in sonicated mitochondria by R21 (n = 3).

(H) COX activity in intact or sonicated mitochondria with or without PDE. HCO₃⁻ stimulation of COX was PDE insensitive in intact mitochondria, whereas it was abolished in sonicated mitochondria by PDE. IBMX prevented the PDE-mediated decrease in COX activity (n = 3).

(I) cAMP levels in sonicated mitochondria were increased by HCO₃⁻ and reduced by PDE. IBMX prevented the decrease in cAMP. Values expressed as pmol cAMP per mg mitochondrial protein (n = 5). Error bars indicate standard deviations. *p < 0.05; **p < 0.001; ***p < 0.0001.
inhibition on mitochondrial respiration were confirmed in whole cells, where KH7 induced a 30% decrease, which was rescued by 8Br-cAMP but not by cytosolic cAMP induced by forskolin + IBMX (Figure 4F, p < 0.0001).

As a second independent method of blocking sAC activity, we used the anti-sAC monoclonal R21antibody, which has inhibitory properties on enzymatic activity (Figure S3). Neither R21 nor nonspecific isotype-matched IgG caused COX inhibition in intact mitochondria. However, R21, but not the IgG control, blocked the bicarbonate-induced increase in COX activity when sAC was made accessible to the antibody by mitochondrial sonication (Figure 4G, p < 0.01).

Third, we measured COX activity in mitochondria treated with bicarbonate in the presence or absence of PDE. In intact mitochondria, bicarbonate stimulated COX activity was unaffected by PDE (Figure 4H). Thus, cAMP was produced in a compartment isolated from external PDE. After sonication, PDE degraded intramitochondrial cAMP (Figure 4I, p < 0.001), and the bicarbonate-induced COX stimulation was abolished (Figure 4H), in an IBMX sensitive manner.

Taken together, this evidence indicates that the sAC-cAMP-PKA signaling pathway is wholly contained within mitochondria and that it modulates OXPHOS in response to physiologically relevant concentrations of bicarbonate.

cAMP-Dependent Phosphorylation of Mitochondrial Proteins
We investigated the pattern of PKA-dependent mitochondrial protein phosphorylation in isolated mitochondria. Proteins were resolved by isoelectric focusing two-dimensional electrophoresis and detected with a PKA substrate-specific anti-phospho Ser/Thr antibody (Bruce et al., 2002; Schmitt and Stork, 2002). Several of the PKA-phosphorylated proteins detectable in untreated mitochondria (indicated by arrows in Figure S4A, upper panel) disappeared or were markedly reduced in the KH7-treated samples (Figure S4A, lower panel).

Many hydrophobic subunits of the respiratory chain are not amenable to isoelectric focusing (unpublished data). Therefore, to examine respiratory chain complexes, we employed 2D-blue-native gel electrophoresis (Schagger and Pfeiffer, 2000; Schagger and von Jagow, 1991). Phosphoproteins were detected using either anti-phospho Ser/Thr antibody (Figure S5A) or the PKA substrate-specific antibody (Figure S5B). Replicate samples were treated with calf-intestinal phosphatase, which abolished most immunoreactive spots on the membrane probed with anti-phospho Ser/Thr antibody, demonstrating the specificity of the antibody (Figure S6). Both anti-phospho Ser/Thr and the PKA substrate-specific antibodies revealed a marked decrease of several phosphoproteins after KH7 or H89. Membranes were reprobed with antibodies against COX subunits, which revealed two of the phosphorylated proteins (denoted by asterisks in the upper panel of Figure S5A) to be COX I and COX IV type II (COX IV-2). The amounts of phosphorylated COX I and COX IV-2 were respectively reduced to 30% and 20% with H89, and to 25% and 5% with KH7.

These results confirm that phosphorylation of several mitochondrial proteins responds to modulation of the mito-sAC signaling pathway and suggest that certain COX subunits are candidates for the regulation of OXPHOS activity.

Physiological Role of the Intramitochondrial Mito-sAC Pathway in the Regulation of OXPHOS and ROS Production
We hypothesized that the physiological role of mitochondrial sAC is to respond to CO2 metabolically generated by the TCA cycle. To test this hypothesis, we measured COX activity in isolated mitochondria “fed” with pyruvate and malate, which fuel the TCA cycle to stimulate CO2 generation. Under these conditions, CAI diminished COX activity by 37% (Figure 5A, p < 0.001), suggesting that the CO2 had to be converted to bicarbonate to sustain COX activity. The inhibitory effect of CAI was reversed by exogenous bicarbonate, which directly stimulates sAC, or by the addition of 8Br-cAMP, which bypasses sAC stimulation. Diminishing CO2 production by retrograde inhibition of the TCA cycle using the complex III blocker antimycin A (AA) reduced COX activity (25%, p < 0.001). As with CAI addition, AA inhibition was rescued by the bicarbonate or 8Br-cAMP (Figure 5A, p < 0.01). Changes in mitochondrial cAMP levels paralleled the changes in COX activity; cAMP decreased in the presence of CAI or AA, demonstrating that TCA cycle-generated CO2 regulates sAC (Figure 5B, p < 0.0001). Finally, OXPHOS was inhibited by CAI also in intact cells, where respiration was diminished by 36% and rescued by 8Br-cAMP (Figure 5C, p < 0.0001).

To investigate the role of the mito-sAC signaling pathway in the physiological adaptation to changes in substrate availability, we cultured cells in medium containing galactose instead of glucose as the main carbon source. Because galactose is utilized in the glycolytic pathway at a much slower rate than glucose, cells are forced to maximize mitochondrial OXPHOS activity to meet their energy needs. Consistently, respiration was increased by 35% in galactose as compared to glucose medium (Figure 5D, p < 0.0001). In contrast to cells grown in glucose, respiration in cells grown in galactose for 48 hr was unaffected by 8Br-cAMP. Presumably, these cells are insensitive to stimulation because OXPHOS is already fully activated by the mito-sAC signaling pathway. Consistently, the decrease in respiration resulting from inhibition of CA, sAC, or PKA was proportionally more effective in galactose than in glucose (46.8% ± 3.97% and 36.5% ± 3.87%, respectively, p < 0.0001).

Activation of the TCA cycle dehydrogenases by increasing mitochondrial calcium (McCormack et al., 1990) with the calcium ionophore A23187 (0.5μM) stimulated respiration in glucose (Figure 5D, p < 0.0001), but not in galactose, because in the latter there was no spare respiratory capacity. Alternatively, calcium could stimulate respiration by uncoupling due to calcium cycling. However, it is unlikely that this mechanism would be sensitive to all three inhibitors of the mito-sAC pathway, which suppress respiration both in glucose and galactose medium. Thus, the only nonconflicting explanation is that the mito-sAC pathway modulates the terminal oxidase of the respiratory chain, which is known to be limiting for respiration in cells (D’Aurelio et al., 2001; Villani and Attardi, 1997; Villani et al., 1998).

Mito-sAC signaling cascade links substrates flux through the TCA cycle with the rate of OXPHOS activity; this linkage could be beneficial to minimize electron leakage from the respiratory chain and prevent ROS generation. Cells grown in galactose produced less ROS than cells grown in glucose (Figure 5E, p < 0.0001), but ROS production in glucose was diminished by...
8Br-cAMP. Addition of 8Br-cAMP to galactose grown cells, whose OXPHOS is already maximally stimulated, had no effect. Consistent with the hypothesis that ROS production depends on the mito-sAC pathway, CAI, KH7, or H89 increased ROS production both in glucose and in galactose (Figure 5E, p < 0.001), and 8Br-cAMP reverted the effects of CAI and KH7 on ROS production, but not those of H89, because its inhibition is downstream of cAMP.

Taken together these results indicate that the mito-sAC pathway is engaged in the adaptation of OXPHOS activity in order to optimize the use of substrates for bioenergetic purposes and, at the same time, minimize ROS production.

**DISCUSSION**

Which are the players involved in the phosphorylation-mediated regulation of OXPHOS function? While many putative mitochondrial phosphoproteins have been identified, most of them have been demonstrated in vitro, making their physiological relevance uncertain. Similarly, the list of kinases and phosphatases identified in mitochondria is constantly expanding (Pagliarini and Dixon, 2006), but their physiological role is still largely unidentified. For example, while both PKA (Livigni et al., 2006; Pagliarini and Dixon, 2006; Prabu et al., 2006; Ryu et al., 2005; Schwoch et al., 1990) and A kinase-anchoring proteins (AKAPs) (Feliciello
et al., 2005; Wang et al., 2001) were found in mitochondria, their substrates and physiological relevance remained controversial. We now show that PKA regulates OXPHOS and that cAMP is generated inside mitochondria in response to metabolically generated CO₂.

We identify sAC as a source of cAMP inside mitochondria. We established the presence and the function of sAC in mitochondria by three independent criteria: (1) sAC is localized inside mitochondria, (2) OXPHOS is decreased by sAC inhibition, and (3) the sAC activator bicarbonate stimulates OXPHOS.

Which are the targets of the mito-sAC pathway? A number of cAMP-dependent phosphorylation events of respiratory chain components have been described. Much work has focused on small subunits of complex I (Papa et al., 1996; Sardanelli et al., 1995; Scacco et al., 2000; Signorile et al., 2002; Chen et al., 2004), although it still remains to be studied whether any of the complex I phosphorylation events are dependent upon the mito-sAC pathway.

Our initial proteomic work has focused on COX, another respiratory chain complex, whose phosphorylation has been studied. COX subunits I, IV-1, and Vb are phosphorylated by PKA in hypoxic cells, resulting in decreased steady-state levels of these proteins and thus COX activity (Prabu et al., 2006). However, changes in COX protein steady-state levels do not occur in our system; therefore, these phosphorylations are unlikely to contribute to the effects described here. Another cAMP-dependent phosphorylation event of COX subunit I occurs on tyrosine residue 304, via PKA regulation of a tyrosine kinase or phosphatase. Tyrosine 304 phosphorylation results in COX inhibition in vitro. However, this tyrosine is on the outer face of the mitochondrial inner membrane (Lee et al., 2005), implying that its phosphorylation should not be dependent upon intramitochondrial cAMP.

cAMP-dependent phosphorylation of subunit I of COX has been postulated to optimize OXPHOS efficiency by keeping the mitochondrial membrane potential low while complex I is fully activated (Bender and Kadenbach, 2000; Kadenbach, 2003). It was also shown that phosphorylation of subunit II of COX by mitochondrial c-Src results in enhanced COX activity in a cAMP-dependent manner (Miyazaki et al., 2003). Either of these mechanisms may be contributing to the increased COX activity described here. COX subunits IV (at amino acid Ser34) and Va (at amino acids Ser4 and Thr35) subunits are phosphorylated in vitro by PKA (Helling et al., 2008). The physiological consequence of these phosphorylation events remains unknown, but because these residues are located on the matrix side of the enzyme (Shinzawa-Itoh et al., 2007), they may be subject to regulation by intramitochondrial PKA.

The mito-sAC pathway is sensitive to metabolic conditions that affect CO₂ production. CO₂ would diffuse throughout a cell, but as it diffuses it encounters discretely localized CA, which speeds up the hydration and dissociation into H⁺ and bicarbonate. Thus, diffusing CO₂ would establish a new equilibrium, with elevated bicarbonate wherever it encounters CA. Such bicarbonate elevation would be transient, as bicarbonate would reach a new equilibrium as a function of the changing local P_CO₂, pH, and buffer capacity. Our data indicate that this occurs within the mitochondrial matrix in response to metabolically generated CO₂. Due to intramitochondrial CA (Dodgson et al., 1980), bicarbonate fluctuates inside mitochondria in direct proportion to the CO₂ generated via the TCA cycle and β-oxidation, and these transient elevations of bicarbonate activate sAC to increase OXPHOS activity and ATP synthesis (Figure 6).

In the well-fed mouse livers used in this study, the mito-sAC pathway appears to be mostly in the "active" state, with high constitutive protein phosphorylation. Thus, we observe a proportionally lower stimulation of OXPHOS by membrane permeable cAMP, bicarbonate, or CO₂ (Figure 1), than downregulation by inhibition of the TCA cycle, carbonic anhydrases, sAC, or PKA (Figures 1 and 4). Physiologically, this pathway mediates OXPHOS activation in response to stimulation of mitochondrial dehydrogenases and utilization of the mitochondrial spare respiratory capacity in cells forced to depend upon OXPHOS for survival in galactose medium. In addition, by providing a mechanism for coupling nutrient utilization with the electron flux, the mito-sAC pathway controls ROS production by the respiratory chain.

With the demonstration that PDE resides inside mitochondria, along with sAC and PKA, we have identified a cAMP microdomain wholly contained within mitochondria. To our knowledge, this represents the first description of a complete signaling cascade inside this organelle. We predict that other intramitochondrial signaling cascades will be identified, which may have evolved in eukaryotes subsequent to endosymbiosis or been retained from primordial bacteria (Margulis and Bermudes, 1985). Interestingly, mammalian sAC is more closely related to

Figure 6. Diagram of the Proposed Free-sAC Regulatory Pathway of OXPHOS

Activators and inhibitors of the various steps of the mito-sAC pathway are indicated. PM, plasma membrane; OM, outer mitochondrial membrane; IMS, inter membrane space; IM, inner mitochondrial membrane; PKA, protein kinase A; sAC, soluble adenylyl cyclase; CA, carbonic anhydrase; KH7, inhibitor of sAC; H89 and PKI (PKI 14-22), inhibitors of PKA; PDE, phosphodiesterase. I through V indicate respiratory chain complexes (I-IV) and complex V (ATPase).
bacterial adenyl cyclases than it is to other mammalian nucleotidyl cyclases (Buck et al., 1999), and its mechanisms of calcium and bicarbonate regulation are conserved in cyanobacteria (Chen et al., 2000; Steegborn et al., 2005).

By linking nutrient metabolism with ATP synthesis and ROS production, the function of the mito-sAC signaling pathway ensures a proper balance between the supply of reducing equivalent to the electron transport chain and their utilization. This feed forward regulation may have profound implications for energy metabolism in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HeLa, human embryonic kidney (HEK293T), and COS-1 cells were grown in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, 1 mM pyruvate, and 4.5 g/L glucose. HEK293T cells expressing sACt were generated by stable transfection as previously described (Hess et al., 2005). COS-1 cells were transiently transfected with a pTurbo plasmid containing sACt cDNA with an HA tag, using FuGene6 (Roche) following the manufacturer’s protocol. Cells were harvested for fractionation and immunoblots after 72 hr.

For galactose growth experiments, HeLa cells were cultured in DMEM without glucose and supplemented with 4.5 g/L galactose, 1 mM pyruvate, L-glutamine, and 10% dialyzed FBS.

Measurements of OXPHOS Function in Cells and Isolated Mitochondria

Intact cells (2 × 10^6) were used for O_2 consumption measurements in an oxygen graph equipped with a Clark electrode, as described (Hofhaus et al., 1996). Mouse liver mitochondria were isolated as described (Fernandez-Vizarra et al., 2002) and state III O_2 consumption driven by specific respiratory chain complexes was measured on 75–100 μg of mitochondrial protein as described (Hofhaus et al., 1996). The calcium ionophore, A23187, was used at 0.5 μM. All reagents were purchased from Sigma-Aldrich.

COX enzymatic activity was measured spectrophotometrically on isolated mitochondrial (2–5 μg of protein) or in cells (2 × 10^6 cells) using a kinetic luminometric assay, as described (Vives-Bauza et al., 2007).

ATP content was measured in isolated mouse liver mitochondria incubated with and without the addition of 3 μM FCCP for 5 min using the Enliten ATP Determination Kit (Promega) as described (Vives-Bauza et al., 2007).

Titration of Agonists and Inhibitors

Each agonist and inhibitor used in this study was titrated to determine conditions resulting in a maximal effect on OXPHOS. All experiments, including those where bicarbonate was added, were performed at constant pH and under ambient CO_2.

In intact cells, titrations of compounds were performed by following mitochondrial O_2 consumption. The concentrations of agonists/inhibitors used were as follows: 88–cAMP (Sigma-Aldrich), 1 mM; H89 (Calbiochem), 1 μM; RpcAMP (Sigma-Aldrich), 25 μM; KH7, 50 μM; 8-CPT-methyl-cAMP (BioMol International), 1 mM; forskolin (Sigma-Aldrich), 10 μM; 3-isobutyl-1-methyl-xanthine (IBMX, Sigma-Aldrich), 50 μM, acetazolamide (carbonic anhydrase inhibitor, CAI, Sigma-Aldrich), 1 μM for 0.5 hr.

Isolated mitochondria were incubated with PKA agonists and inhibitors in MAITE medium (10 mM Tris-HCl, pH 7.4; 2.5 mM succrose; 75 mM sorbitol; 100 mM KCl; 10 mM K_2HPO_4; 0.05 mM EDTA; 5 mM MgCl_2; 1 mg/ml BSA) in the presence of a cocktail of phosphatase inhibitors (Sigma-Aldrich). When bicarbonate was used to stimulate sAC, MAITE containing 300 mM Tris-HCl was used to buffer the pH. Controls for bicarbonate were also run in MAITE 300 mM Tris-HCl. The following conditions were used for all experiments: 1 mM 8Br-cAMP, 1 μM H89, 25 μM KH7, and 30 mM bicarbonate for 10 min. Other agonist/inhibitors used in this study were as follows: cAMP (Sigma-Aldrich), 1 mM; 8-CPT-methyl-cAMP, 1 mM; forskolin, 10 μM + IBMX 50 μM; myristoylated protein kinase inhibitor peptide 14-22 (PKI 14-22, Sigma-Aldrich), 1 μM; KH7.15 25, 1 μM acetazolamide (CAI), 1 μM.

Membrane Potential Measurements

Mitochondrial membrane potential (ΔΨ_m) was measured in enriched mouse mitochondrial fractions using the dye Safranin-O as previously described (Kwong et al., 2007). ΔΨ_m was calculated based on the linear response of the dye Safranin-O in the region of our measurements (50–170 mV), corresponding to the difference between the maximal fluorescence induced by the substrates and the minimum fluorescence determined by the inhibitors (Akerman and Wikstrom, 1976; Nedergraard, 1983; Zanotti and Azzone, 1980). Changes in ΔΨ_m were expressed as a percentage of the untreated mitochondria.

Mitochondrial Purification by Nycodenz Gradient

Crude mitochondria from mouse liver (5 mg) or cultured cells (1 mg) were resuspended in 3.3 ml of 25% Nycodenz solution (60% stock; Gentaur) diluted in 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4. Resuspended mitochondria were applied to a discontinuous Nycodenz gradient composed of the following layers: 1.6 ml of 40%, 1.6 ml of 34%, 2.3 ml of 30%, 3.3 ml of resuspended mitochondria, 2.3 ml of 23%, and 0.7 ml of 20% Nycodenz. Samples were centrifuged at 95,000 g for 2 hr in a SW41Ti Sorvall Rotor, and the following fractions were collected: fraction 2 between 20%–23%, fraction 3 between 23%–25%, fraction 4 between 25%–30% and fraction 5 between 30%–34%. Fraction 4 from mouse liver mitochondria was subjected to a second round of Nycodenz gradient fractionation and fractions 2, 3, 4, and 5 (denoted as 4.2, 4.3, 4.4, and 4.5, respectively) were collected.

Mitoplast Preparation

Isolated mitochondria 500 μg (1 mg/ml) were resuspended in MS-EGTA (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4). Water (1/10 volume) and digitothin (1 mg digitothin/5 mg mitochondrial protein) were added, and the mixture was incubated on ice for 45 min. Then, KCl (150 mM) was added, followed by incubation for 2 min on ice and centrifugation at 18,000 g for 20 min at 4°C. The pellet containing the mitoplasts fraction was resuspended at 1 mg/ml in 300 mM Tris-HCl, 10 mM CaCl_2, pH 7.4. The supernatant containing the postmitoplasts fraction was precipitated with 12% TCA and centrifuged at 18,000 g for 15 min at 4°C. The pellet was resuspended in 500 μl acetone and centrifuged at 18,000 g for 15 min at 4°C.

Determination of Phosphodiesterase Activity in Isolated Mitoplasts

Mitoplasts were sonicated, and 50 pmol of cAMP was added to each sample. IBMX (50 μM) or K_7 (25 μM) were used to prevent degradation and synthesis of cAMP, respectively. Samples containing 50 pmol cAMP, 0.1 unit of phosphodiesterase (PDE, Sigma-Aldrich), and 10 units of calmodulin (Sigma-Aldrich), with or without IBMX (but no mitoplasts) were used as controls. Reactions were performed at 30°C for 30 min.

Immunoblot Analyses

For western blot analyses of COX subunits in crude mitochondria, 10 μg of protein were separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto PVDF filters (BioRad).

For protease protection assays 20 μg of mitochondrial protein from COS cells expressing sACt-HA were treated with 20 μg/ml PK for 20 min on ice. Then, PK was inactivated with 2 mM phenylmethylsulfonylfluoride for 10 min on ice. Prior to PK treatment, one aliquot of mitochondria was solubilized with 1% Triton X-100 (Sigma-Aldrich) for 15 min on ice.

For isoelectric focusing of mitochondrial samples, 100 μg of protein were processed with a Ready Prep 2D Cleanup kit (BioRad) and resuspended in 125 μl of rehydration buffer (BioRad). Samples were applied to 3–10 IPG strips (BioRad) and incubated overnight at room temperature. Isoelectric focusing and 2D SDS-PAGE were run under standard conditions, and proteins were transferred onto a PVDF filters.

For blue native (BN) 2D gels (Schagger and von Jagow, 1991), 50–75 μg mitochondrial protein were applied on a 5%–13% gradient BN gel followed by a 12.5% 2D denaturing gel. After electrophoresis, proteins were electroblotted onto PVDF filters and sequentially probed with specific antibodies.

274 Cell Metabolism 9, 265–276, March 4, 2009 ©2009 Elsevier Inc.
Replicate samples were treated with calf-intestinal phosphatase for 1 hr at 37°C to dephosphorylate mitochondrial proteins. For protein detection, the following antibodies were used: R21 against human sAC (Zippin et al., 2003), PKA (Millipore), phospho Ser/Thr residues, Tim 23 (BD Transduction Laboratories), phospho Ser/Thr PKA substrate-specific (Cell Signaling Technology), COX I, COX IV-2, GAPDH, and VDAC (Invitrogen), PDI, Hsp60 and cytochrome c (Stressgen), HA (AbCam), and Tom 40 (Santa Cruz Biotechnology).

**Phosphodiesterase and Anti-sAC Antibody Treatment of Isolated Mitochondria**
Isolated crude mouse liver mitochondria (500 μg), either intact or sonicated with three pulses of 2 s each at half of maximal frequency in a Branson Sonifier 250 sonicator (VWR Scientific), were resuspended in 300 mM Tris-HCl, 10 μM CaCl2, pH 7.4 (1 mg/ml final concentration), and incubated with or without 0.1 unit of phosphodiesterase at 30°C for 30 min. Ten units of calmodulin, a PDE cofactor, were added to all samples. Bicarbonate (30 mM) and IBMX (50 μM) were added to stimulate sAC or inhibit PDE, respectively. For immunoinhibition of sAC, samples were treated with 0.5 μg of the R21 anti-sAC antibody or with mouse IgG subtype K (Sigma). Phosphatase inhibitors were used during the incubations.

**Titration of R21 Antibody**
Cyclase assays were performed in 100 μl volume with equivalent amounts of total protein in the presence of 50 mM Tris (pH 7.5), 20 mM creatine phosphate, and 100 U/μL CPK where required, substrate 2.5 mM [32P]ATP and 10 mM MgCl2. Reactions were incubated at 30°C for 30 min, and stopped with 200 μl 2% SDS, [32P]cAMP was recovered using a two-column method as described in (Salomon, 1979). Kinetic analysis was performed using GraphPad Prism software.

**α-Ketoglutarate Dehydrogenase Complex Activity Treatment of Isolated Mitochondria**
α-ketoglutarate dehydrogenase complex (KGDH) activity was performed in the oxgraph in respiration medium containing cocarboxilase (TPP, Sigma-Aldrich), 0.3 mM β-nicotinamide adenine dinucleotide (β-NAD*, Sigma-Aldrich), 0.5 mM coenzyme A (Sigma-Aldrich), 0.24 mM α-ketoglutarate (Sigma-Aldrich), 5mM dithiothreitol (DTT, Sigma-Aldrich), 1 mM; and 0.5 units of KGDHC (Sigma-Aldrich). After 3 min reaction, isolated crude mouse mitochondria (100 μg) were added to the medium and TMPD/ascorbate-dependent respiration was measured after blocking complex III with antimycin A as described above. Control samples were run in the same conditions, but in the absence of the KGDHC enzyme.

**cAMP Measurements**
cAMP measurements were performed according to manufacturer’s instructions using the Direct Correlate-EIA cAMP Kit (Assay Designs, Inc.). One hundred microliters of sample were measured. If necessary, samples were diluted to bring the CAMP level of the sample within the linear range of the assay.

**ROS Production Determination**
Cells were grown in glucose or galactose medium for 48 hr in a 96-well plate and then incubated with the different compounds for 2 hr at the same concentrations used for the other biochemical assays. Cells were incubated with 2',7'- dichlorofluorescein diacetate (H2-DCFDA; Invitrogen, 10 μM MgCl2. Reactions were incubated at 30°C for 200 μl volume with equivalent amounts of total protein in the presence of 50 mM Tris (pH 7.5), 20 mM creatine phosphate, and 100 U/μL CPK where required, substrate 2.5 mM [32P]ATP and 10 mM MgCl2. Reactions were incubated at 30°C for 30 min. Ten units of calmodulin, a PDE cofactor, were added to all samples. Bicarbonate (30 mM) and IBMX (50 μM) were added to stimulate sAC or inhibit PDE, respectively. For immunoinhibition of sAC, samples were treated with 0.5 μg of the R21 anti-sAC antibody or with mouse IgG subtype K (Sigma). Phosphatase inhibitors were used during the incubations.

**Statistical Analyses**
Comparisons between groups were made using one-way ANOVA. Pair-wise comparisons were made by post hoc Fisher PLSD test. Differences were considered statistically significant at p < 0.05. Data analyses were performed using the statistical program StatView. In all experiments, error bars indicate standard deviations (Adept Scientific, UK).
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